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Original article

Longitudinal study of ICET-A on glucose tolerance, insulin sensitivity and β-cell secretion in eleven β-thalassemia major patients with mild iron overload

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Abstract. *Background:* Iron chelation therapy (ICT) is the gold standard for treating patients with iron overload, though its long-term effects are still under evaluation. According to current recommendations regarding transfusion-dependent (TD) β-thalassemia major (β-TM) patients, their serum ferritin (SF) levels should be maintained below 1,000 ng/mL and ICT should be discontinued when the levels are <500 ng/mL in two successive tests. Alternatively, the dose of chelator could be considerably reduced to maintain a balance between iron input and output of frequent transfusions. *Study design:* Due to the paucity of information on long-term effects of ICT in β-TM with low SF levels on glucose homeostasis, the International Network of Clinicians for Endocrinopathies in Thalassemia and Adolescence Medicine (ICET-A) promoted a retrospective and an ongoing prospective observational study with the primary aim to address the long-term effects of ICT on glucose tolerance and metabolism (β-cell function and peripheral insulin sensitivity) in adult β-TM patients with persistent SF level below 800 ng/mL. *Patients and Methods:* 11 β-TM patients (mean age: 35.5 ± 5.5 years; SF range: 345-777 ng/mL) with normal glucose tolerance test (OGTT) or abnormal glucose tolerance (AGT) for a median of 5.3(1.1-8.3) years. *Results:* Abnormal glucose tolerance (AGT) was observed in 7 patients (63.6%) at first observation and) persisted in 6 patients (54.5%) at last observation. None of them developed diabetes mellitus. AGT was reversed in two patients. One patient with NGT developed early glucose intolerance (1-h PG ≥155 and 2-h PG <140 mg/dL). Three out of 5 patients with isolated impaired glucose tolerance presented a variation of ATG. Stabilization of low indices for β-cell function and insulin sensitivity/resistance was observed. One patient developed hypogonadotrophic hypogonadism. Three out of 6 patients with SF below 500 ng/dL had hypercalciuria. *Conclusion:* Despite low SF level, the burden of endocrine complications remains a challenge in β-TM patients. The ability to keep iron at near "normal" level with acceptable risks of toxicity remains to be established. (www.actabiomedica.it)

Key words: β-thalassemia major, glucose homeostasis, insulin sensitivity, β-cell secretion, serum ferritin, follow-up

Introduction

The standard therapy for transfusion-dependent (TD) β-thalassemia major (β-TM) is to transfuse every two- three weeks appropriate amount of leucocytes-depleted packed blood red cells (PBRCs) to maintain a pre-transfusion hemoglobin (Hb) level > 9.0 g/dL and post-transfusion Hb below 14–15 g/dL, in order to provide efficient oxygen to tissue and suppress bone marrow hyperactivity activity (1). Since each unit of PRBCs contains 200–250 mg iron, transfusion therapy progressively increases iron stores to many times the normal range if untreated.

In the process of iron overload (IOL), iron initially accumulates in hepatic and splenic macrophages which recycle transfused red blood cells once they become senescent (1-3). When hepatic storage capacity is exceeded, the iron binding capacity of plasma transferrin is often surpassed, with concomitant generation of non-transferrin-bound iron (NTBI). Persistently high plasma NTBI levels can lead to uncontrolled ingress of labile plasma iron (LPI) into cells, forming highly reactive oxygen free radicals, causing peroxidation of membrane lipids and oxidative damage to cellular proteins in many organs such as liver, spleen, bone marrow, pancreas, heart, pituitary and central nervous system and other endocrine glands (3-5). Although the transport mechanism for the transport of NTBI into cells remains unclear, it is evident that selected tissues are particularly susceptible to excess iron uptake when NTBI is present. Endocrine glands and the heart almost exclusively absorb NTBI (in its various species), whereas in the liver iron uptake is predominantly transferrin-mediated (3). NTBI uptake into endocrine glands depends on L-type Ca^{2+} channels, which are known to be present in large numbers in pancreatic beta cells and in various cell types of the anterior pituitary gland (gonadotrophs, thyrotrophs, corticoptrophs) and in the parathyroid gland (5). The pathologic factors accountable for variable organ-specific iron loading remain unclear. Cardiac iron accumulation is markedly delayed compared with other organs, as liver and pancreas (4).

The high prevalence of hypogonadotropic hypogonadism (HH), thalassemia-related diabetes mellitus (TR-DM), and cardiomyopathy suggest that anterior pituitary, cardiac myocytes, and pancreatic β-cells are particularly susceptible to iron toxicity (4-7). Abnormal glucose tolerance (AGT) increases progressively with age, starting from the age of 11 years (8) and even earlier in patients with severe IOL (4-7). Therefore, it is important to detect "silent" glucose abnormalities before the development of TR-DM.

Current approaches to quantify iron overload and monitor iron homeostasis are: assessment of SF and transferrin saturation as well as assessment of liver iron content (LIC) and myocardial iron, as determined by magnetic resonance imaging (MRI) (1-3).

Recently, a SF level of 841 ng/mL was detected as a significant threshold level for severe pancreatic iron burden, assessed by pancreatic MRI, with 83% sensitivity and 54% specificity (P = 0.04) (9). Thus, early diagnosis and noninvasive tests for monitoring of treatment of IOL are essential to optimize the management of iron loading.

Currently, three iron chelators are available on the market for the treatment of IOL: deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX). They differ in the route and starting time of administration as well as side effect profiles (1,2). The goals of ICT treatment are to prevent and remove the excess of iron from the body and to reduce plasma and cytosolic levels of reactive labile iron (Fe^{+2};NTBI/LPI), as quickly as possible and to remove the excess iron from the body. Monotherapy is generally utilized if the iron burden is at acceptable or near-acceptable levels and the dose is adjusted accordingly. A combination of iron chelators is employed for patients with high iron burden, iron-related organ injury, or where adverse effects of chelators preclude the administration of an appropriate chelator dose (1,2).

Generally, it is recommended that in β-TM patients SF levels should be maintained below 1,000 ng/mL and when the levels are <500 ng/mL, in two successive tests, iron chelation therapy (ICT) should be discontinued to avoid adverse events due to overzealous ICT (1). Alternatively, the dose of chelator could be reduced considerably to maintain a balance between iron input and output from the frequent transfusions.

The primary recommended screening approach for AGT remains the oral glucose tolerance test (OGTT). Its use is widely advocated with the recommendation

that the test should be carried out, preferably combined with the determination of insulin secretion, initially at 10, 12, 14, and 16 years and annually thereafter (10).

Due to the paucity of information on the long-term effects of ICT on glucose homeostasis in β-TM patients with SF levels below 1,000 ng/mL (11-13), the International Network of Clinicians for Endocrinopathies in Thalassemia and Adolescence Medicine (ICET-A) promoted a retrospective and an ongoing prospective observational study with the primary aim being to address the long-term effects of ICT on glucose metabolism in adult β-TM patients with persistent low SF levels (below 1,000 ng/mL) and to gain insights the effects of ICT on β-cell function (first-phase insulin secretion) and peripheral insulin sensitivity (hepatic and peripheral tissue sensitivity to insulin) in response to a glucose load.

Study design at first (baseline) evaluation

From October 2010 to October 2022, twenty-four β-TM patients with persistent low SF levels (<1,000 ng/mL) were referred for consultation or second opinion, basically for endocrine and glucose disorders, to a single Italian centre, experienced in Endocrinopathies of Thalassemias (Pediatric and Adolescent Outpatient Clinic, Private Accredited Quisisana Hospital, Ferrara, Italy). All had been subjected to OGTT according to the international recommendations (10).

At baseline, 14/24 β-TM patients (58.3%) had SF below 500 ng/mL, and the remaining between 500 and 1,000 ng/mL. Cardiac and LIC assessed by MRI T2* were normal in all but two patients. Substantially, the study showed that efficient iron chelation monotherapy did not entirely prevent the development of glucose and insulin (secretion and sensitivity) disorders and the development of an additional case of HH (14).

Follow-up study

In order to obtain further information on the course of the evolution of glucose tolerance, centers that had referred patients were contacted in September 2022. A detailed explanation of the nature and purpose of the diagostic OGTT follow-up survey was given. Physicians of thalassemia centers were also required to report, before OGTT follow-up study, the following available information and data: mean pre-transfusional Hb level and SF (ng/mL) in the last year, liver and renal function, serum calcium, phosphate, alkaline phosphatase, urine analysis, thyroid function, basal cortisol, luteinizing hormone and follicular-stimulating hormone (LH and FSH), estradiol (in females), testosterone (in males), and relevant organs imaging (cardiac, pancreatic and liver MRI T2*), if done in the last 18 months.

Eleven β-TM patients with a follow-up of at least one year, respect to the first evaluation, spontaneously agreed to participate in the OGTT re-evaluation.

The patients received appropriate instructions prior the OGTT test regarding exercise, stress, sleep, smoking, hydration status, and consumption of caffeine, and carbohydrates. They were also advised to have at least 3 days of "unrestricted diet" (moderate- to high carbohydrate intake), containing >150 grams of carbohydrates daily prior to the OGTT test (15,16).

Clinical re-evaluation and OGTT

The re-evaluation consisted of a comprehensive medical history and full physical examination. Moreover, patients received a detailed explanation on the purpose of OGTT and the potential benefits for re-assessing glucose homeostasis, insulin secretion and sensitivity (7,8,10-12).

A standard OGTT (max 75 g of glucose in 250–300 mL water) was performed in the morning, after an overnight fast, in subjects clinically stable and without a history of acute infection in the previous 3 weeks. Venous blood samples were collected at 0, 30, 60, 90, 120 and 180 min, after glucose load, for plasma glucose (PG) and insulin assay,

Glucose tolerance was classified in accordance with the American Diabetes Association criteria (17). Early intolerance [early glucose intolerance (EGI)] was diagnosed when 1-h PG was ≥155 and 2-h PG >140 mg/dL (18). The term "abnormal glucose tolerance" (AGT) was used to define isolated impaired fasting glucose (IFG), EGI, and impaired glucose tolerance (IGT), but not diabetes.

Calculations of variables derived from OGTT

To evaluate the insulin secretion, the following indices were calculated: Early-phase insulin secretion index (IGI: Δ 0-30 insulin/Δ 0-30 glucose min) (19) and the homeostasis model assessment of β-cell function index [HOMA-β: Insulin x 360/glucose (mg/dl)-63] (20).

The following indices were used for the determination of insulin sensitivity/ resistance: Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), using the formula: [fasting glucose (mg/dL)] × [fasting insulin (μU/mL)]/405 (21), and Matsuda index (MI) calculated as:10,000/square root of [fasting glucose (mg/dL) × fasting insulin (μU/mL)] × [mean glucose (mg/dL) × mean insulin (μU/mL), which encompasses both hepatic and peripheral tissue insulin sensitivity (22,23).

Moreover, oral disposition index (oDI) was calculated by the product of insulin secretory capacity (IGI) and insulin sensitivity (MI). This index provides a measure of β-cell function adjusted for insulin sensitivity and has been shown to be predictive of development of diabetes in the general population (24); this index was confirmed by us in 25 β-TM patients with diabetes mellitus (mean oDI: 1.08 ± 0.73 vs. 4.89 ± 2.9 in 12 β-TM patients with normal glucose tolerance test and 13.8 ± 10.1 in 16 healthy controls, P = < 0.0001) (8).

Glucose and insulin areas under the curves were also calculated from 0 to 180 min (AUC_{0-180}) of the OGTT using the trapezoidal method. Total insulin secretion was calculated as the ratio of incremental areas under the insulin and glucose curves during the OGTT ($AUC_{Insulin\ 0-180}$ / $AUC_{Glucose\ 0-180}$). Finally, the ratio insulin at 0 min/insulin at 120 min, during OGTT, was also calculated. Higher insulin levels at 120 min vs. 30 min indicate a significant dysregulation of glucose metabolism (21).

Methods for analytical assays

Plasma glucose was assayed by an automated glucose oxidase method and expressed in mg/dL.

Insulin was measured using the Immulite immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). The analytical sensitivity was 2 μIU/mL.

SF levels were measured using Beckman Coulter DXL 800 automatic chemiluminescence immuno-analyzer. SF assay was calibrated against the Third International Recombinant Standard for ferritin (National Institute for Biological Standards and Control Code 94/572). The 90th percentile of reported normal values in females and males are 201 and 243 ng/mL, respectively (25).

Statistical analysis

All numeric variables were expressed as mean ± standard deviation (SD). Comparison of different variables in the two groups was made using unpaired student t-test and Mann-Whitney test for normal and non-parametric variables, respectively. Continuous variables were also compared using a one-way analysis of variance (ANOVA). Chi-square ($\chi 2$) test was used to compare the frequency of qualitative variables among the different groups. Pearson's and Spearman's correlation tests (2-tailed) were used to study correlations between variables with parametric and non-parametric distributions, respectively. A P-value < 0.05 was considered statistically significant. For the statistical analysis, a software program was used and validated, according to Alder and Roesser (26).

Ethics

The re-evaluation was conducted in accordance with the Declaration of Helsinki. Participants were informed that the OGTT reassessment was voluntary and the collected information would remain confidential. Ethics Committee approval was not required because patients underwent only routine annual OGTT according to current literature (7,8,10-12). Moreover, the glucose homeostasis test was non-interventional, there was no risk of physical harm to patients, an anonymized dataset was analyzed, and informed patient's assent was obtained at the time of data collection (27).

Results

a. Subjects

The study group consisted of 11 β-TM patients and 11 healthy adult controls. The mean age of β-TM patients at last observation was 35.5 ± 5.5 years (range 25.5–42.5 yrs). All were receiving standard therapy, with regular transfusions of packed red blood cells and iron chelation therapy.

The mean age of 11 healthy adult volunteers was 23.8 ± 3.2 years. None was carrier for β-thalassemia or overweight/obese (28).

Before admission to follow-up OGTT, the modalities employed by different centers to screen glucose homeostasis were 2-h OGTT (every 18-24 months) in 3 patients (27.2%) or PG determination at baseline and 2-h after glucose load (3 patients, 27.2%). In the remaining 5 cases, venous or capillary blood glucose levels were checked before blood transfusions or 2-h after meal using a glucometer.

Demographic and biochemical characteristics of the study population are reported in Table 1.

Among the different parameters that were assessed no statistically significant difference was found after 5.5 ± 2.2 years (median 5.3 year; range:1.1-8.3 years) of

Table 1. Demographic characteristics, biochemical and diagnostic parameters of 11 β-TM patients at first and last observation.

Variables	At first observation	At last observation	P value
Number of β-TM patients	11	11	-
Age (yr)	30.7 ± 4.9	35.5 ± 5.5	-
Sex (M/F)	6/5	6/5	-
Body Mass Index (kg/m2)	21.3 ± 1.8	21.8 ± 2.0	NS
Splenectomy	8/11	8/11	NS
Mean pre-transfusional Hb level (g/dL) in the year before first and last observation	8.93 ± 0.48	9.10 ± 0.37	NS
Iron chelation therapy at OGTT: **Desferrioxamine (DFO) (n)** **Deferiprone (DFP) (n)** **Deferasirox (DFX) (n)**	- 8 2 1	- 8 2 1	- - - -
Mean serum ferritin (ng/mL) in the last year. Mean, SD and range	424.1± 151.8 229-837	524.4 ± 146.3 345-777	N.S. -
Family history of diabetes: **None** **Yes (n. and %)**	 9 2 (18.1%)	 8 3 (27.2%)	 - N.S.
ALT (normal values: < 33 IU/L)	26.4 ± 10.9	26.6 ± 9.2	NS
MRI LIC (mg/g dry weight) ^	1.52 ± 0.67	See text	-
MRI global myocardial T2* (ms) ^^	33.55 ± 10.76	See text	-
Growth and endocrine complications: **1. Short stature (≤ 3rd centile) (n and %)** **2. HH (n and %)** **3. Acquired secondary HH (n and %)** **4. Central HT (n and %)** **5. Hypoparathyroidism (n and %)** **6. Hypocortisolism (n and %)**	- 4 (36.3%) 2/11 (18.1%) 6/11 (54.5%) 0 0 0	- 4 (36.3%) 2/11 (18.1%) 7/11 (63.6%) 0 0 0	- - - NS - - -

Abbreviations= = **N:** number; **ALT:** alanine aminotransferase**; MRI:** magnetic resonance imaging; **LIC**: liver iron concentration; **HH:** hypogonadotropic hypogonadism; **Central HT:** Central hypothyroidism; **NS:** not significant. **(^) MRI LIC** values were expressed as mg/g/dry weight and classified into: normal (LIC <3 mg Fe/g dry weight); mild (LIC: > 3 and < 7 mg Fe/g dry weight), moderate (LIC: ≥ 7 and < 15 mg Fe/g dry weight) and severe overload (LIC: ≥ 15 mg Fe/g dry weight). **A cardiac T2*** measurement of ≥ 20 ms was taken as a "conservative" normal value forglobal values.

observation. At first observation, all but two β-TM patients had a SF level < 500 ng/mL (total mean SF level: 341.1 ± 64.9 ng/mL vs. 790 and 548 ng/mL), and at the last observation a SF level < 500 ng/mL was found in 6 β-TM patients (total mean SF level: 406.0 ± 51.1 vs. 666.6 ± 83.1 ng/mL in the remaining 5 patients; P = 0.0001).

At last observation, patients with SF below 500 ng/mL were on therapy with DFO (20-30 mg/kg/day, 5-6 times a week, administered subcutaneously by pump during the night over a span of 8–10 hours, 2 patients were on monotherapy with DFP (50-60 mg/kg/day.) and 1 patient on monotherapy with DFX tablet for oral suspension (14 mg/kg/day).

A liver and cardiac MRI T2* was assessed, in the last 18 months, only in 2 patients. In both cases the liver iron concentration (LIC) and global cardiac T2* were in the normal range (Table 1, see abbreviations: for parameters of normality). No significant abnormal biochemical or hormonal parameters documented before 3-h OGTT. Three out of 6 patients with SF below 500 ng/dL had a severe hypercalciuria (urinary calcium from 12 to 18 mg/kg/day; normal values: < 4 mg/kg/day), two were on treatment with DFO and 1 with DFX. In two patients, this was associated with microscopic hematuria (4–6 red blood cells per high-power field) and in one patient to mild proteinuria (30 mg/dL) detected by one time simple dipstick test.

An additional case of acquired hypogonadism, in a 39.9 years old male patient with SF of 369 ng/mL and low gonadotrophins and testosterone levels, was documented 7.8 years after the first observation. The early morning (8.30-9:00 AM) measurement of serum testosterone was 180 ng/dL.

b. Categorization of OGTT at last observation and sequential changes in plasma glucose

An abnormal glucose tolerance (AGT: isolated IFG, EGI, IGT, IFG+IGT) was observed in 7 (63.6%) β-TM patients at first observation and in 6 patients (54.5%; P = 0.67) at last observation. None of them developed diabetes. Two patients with AGT became NGT and one patient with NGT developed EGI. In 3 out of 5 patients with isolated IFG (PG at 0' time of OGTT:112-115 mg/dL) glucose homeostasis worsened. The detailed evolution of glucose tolerance abnormalities in 11 β-TM patients is illustrated in figure 1.

Fasting PG as well as plasma glucose response during OGTT was significantly higher (except for fasting PG value at baseline) compared to controls. At all times no significant statistical differences were documented between PG values at first and last observation (Figure 2).

PG area under the curve ($AUC_{Glucose\ 0\text{-}180}$) was statistically different in β-TM patients compared to controls (P= 0.0001). Interestingly, the shape of PG curve in β-TM patients at first observation showed a delayed PG peak (> 30 min) during OGTT compared to last observation. However, this pattern of PG at 30 minutes during OGTT was not statistically significant (Table 2 and Figure 3).

c. Insulin secretion and sensitivity indices, and sequential insulin changes

A detailed analysis of insulin levels during OGTT, the $AUC_{Insulin\ 0\text{-}180}$ (μU/mL) and the surrogate β-cell function indices of β-TM patients, at first and last observation, compared to controls are shown in table 2. The glucose curve shape β-TM patients was classified as 'monophasic' (PG increased after an oral glucose load between 30 and 90 min until a peak was reached, followed by a subsequent decline of ≥ 4.5 mg/dL) (Figure 3) (29).

The ratio of insulin at 30 minute/insulin at 120 minutes was not statistically different between the baseline and last observation (1.13 ± 0.54 vs. 1.48 ± 0.94; P=0.29). In particular, a ratio below 1 was found in 5 β-TM patients at baseline and at last observation. Higher insulin levels at 120 min vs. 30 min indicate a significant dysregulation of glucose metabolism (21).

Abnormalities of glucose tolerance were associated with a significantly attenuated IGI (first phase of insulin response), which is considered to be the earliest sign of glucose intolerance and impaired oral disposition index (oDI). The latter index reflects the relationship between β-cell function (first-phase insulin secretion) and peripheral insulin sensitivity (hepatic and peripheral tissue sensitivity to insulin). Interestingly, at last observation, IGI, HOMA-β and oDI very also lower in 5 β-TM patients with normal glucose tolerance compared to controls.

A modest correlation was found with IGI [IGI: 0.59 ± 0.19 vs. 1.54 ± 0.99 (P = 0.055)] and a

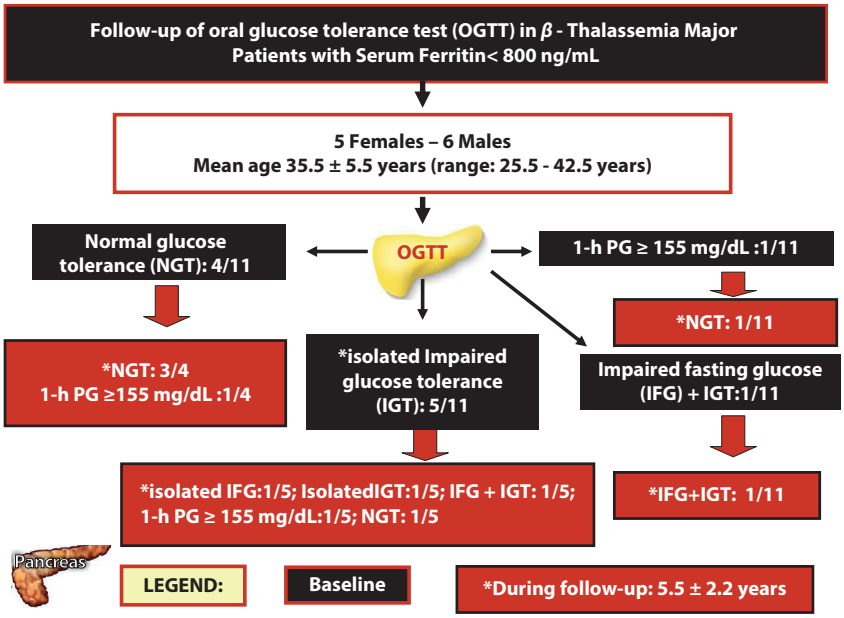

Figure 1. Follow-up of oral glucose tolerance test (OGTT) in β-thalassemia major patients with serum ferritin below 800 ng/dL at baseline and last evaluation. The frames in black refer to first observation (baseline) and in brick colour at last observation. Isolated impaired fasting plasma glucose (PG) is defined as levels of ≥ 100 to 125 mg/dL (≥ 5.6 to 6.9 mmol/L) in fasting patients. Impaired glucose tolerance is defined as 2-h PG levels of 140 to 199 mg per dL (7.8 to 11.0 mmol/L).

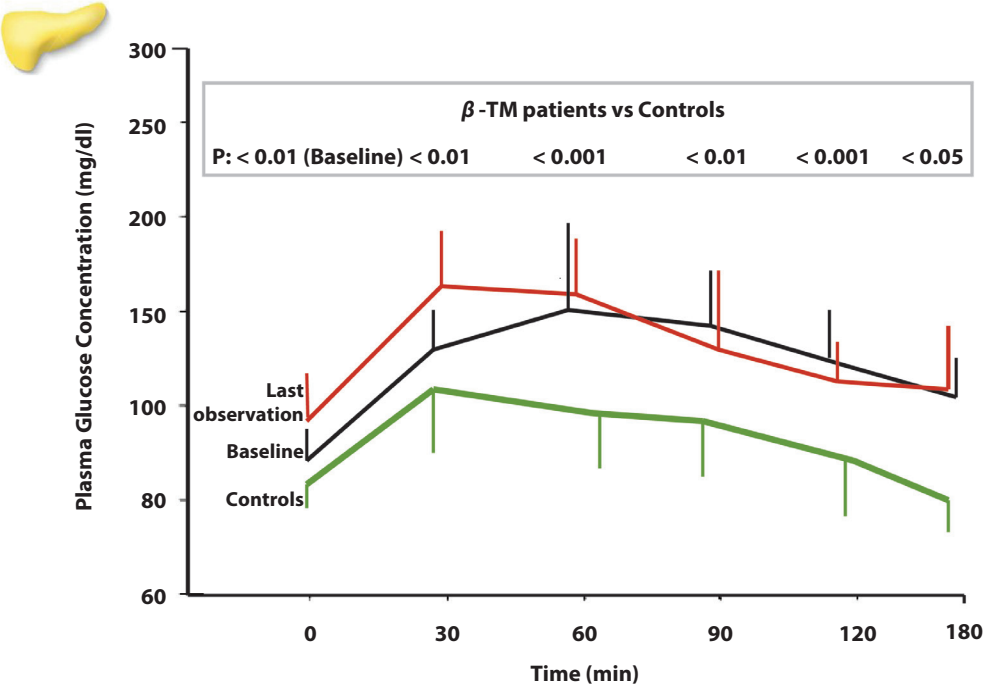

Figure 2. Profiles of plasma glucose during oral glucose tolerance test in β-TM patients, at first and follow-up evaluations, and in controls.

Table 2. Oral glucose tolerance test and derived indices of insulin sensitivity/resistance, secretion and β-cell function at first and last observation in 11 β-TM patients versus controls.

Variables	At first observation (11 β-TM patients)	At last observation (11 β-TM patients)	Controls (11 subjects)
Fasting plasma glucose (mg/dL)	91.0 ± 12.3	97.5 ± 10.2 (§)	84.6 ± 7.2 (§; vs. first obs.; ** vs. last obs.)
30 min glucose (mg/dL)	143.7 ± 20.7	158.0 ± 26.9 (§)	118.8 ± 18.8 (**)
1-h glucose (mg/dL)	150.8 ± 36.4	155.8 ± 35.6 (§)	95.4 ± 18.0 (***)
2-h glucose (mg/dL)	135.6 ± 31.5	128.5 ± 24.9 (§)	84.6 ± 18.0 (***)
3-h glucose (mg/dL)	101.2 ± 27.2	103.1 ± 28.4 (§)	77.4 ± 14.4 (*)
Glucose-Peak (mg/dL)	159.6 ± 26.9	171.8 ± 30.5 (§)	118.8 ± 18.8 (***)
$AUC_{Glucose\ 0\text{-}180}$ (mg/dL)	492.8 ± 72.6	505.9 ± 83.4 (§)	337.5 ± 58.3 (****)
Fasting insulin (μU/mL)	5.6 ± 2.2	5.1 ± 1.7 (§)	7.0 ± 3.0 (§)
30 min insulin (μU/mL)	36.5 ± 38.4	38.4 ± 20.7 (§)	46.2 ± 25.3 (§)
1- h insulin (μU/mL)	42.5 ± 26.3	47.8 ± 26.2 (§)	37.0 ± 17.6 (§)
2 -h insulin (μU/mL)	34.2 ± 17.2	30.8 ± 14.6 (§)	24.1 ± 12.2 (§)
3- h insulin (μU/mL)	17.1 ± 7.0	17.0 ± 12.7 (§)	10.7 ± 7.7 (§)
Insulin- Peak (μU/mL)	45.0 ± 13.9	55.9 ± 23.8 (§)	46.2 ± 25.3 (§)
$AUC_{Insulin\ 0\text{-}180}$ (μU/mL)	119.0 ± 59.2	129.6 ± 58.3 (§)	92.9 ± 44.8 (§)
Insulinogenic index (IGI)	0.59 ± 0.30	0.56 ± 0.33 (§)	1.54 ± 0.99 (**)
$AUC_{Insulin\ 0\text{-}180}/AUC_{Glucose\ 0\text{-}180}$	0.23 ± 0.10	0.25 ± 0.11 (§)	0.27 ± 0.09 (§)
HOMA-IR	1.26 ± 0.55	1.24 ± 0.50 (§)	1.16 ± 0.76 (§)
HOMA- β	62.4 ± 21.3	55.9 ± 13.9 (§)	120.0 ± 21.4 (***)
Matsuda- IR index (0-120)	7.37 ± 2.48	7.23 ± 2.61 (§)	8.71 ± 2.85 (§)
Oral Disposition Index (oDI)	4.26 ± 2.49	3.82 ± 2.28 (§)	12.11 ± 6.55 (***)

Abbreviations= AUC: area under the curve; β-TM patients comparison at first and last observation, = **(§):** N.S; β-TM patients comparison at first and last observation vs. Controls = **(*):** < 0.05; **(**):** < 0.01; **(***):** < 0.001; (****): < 0.0001.

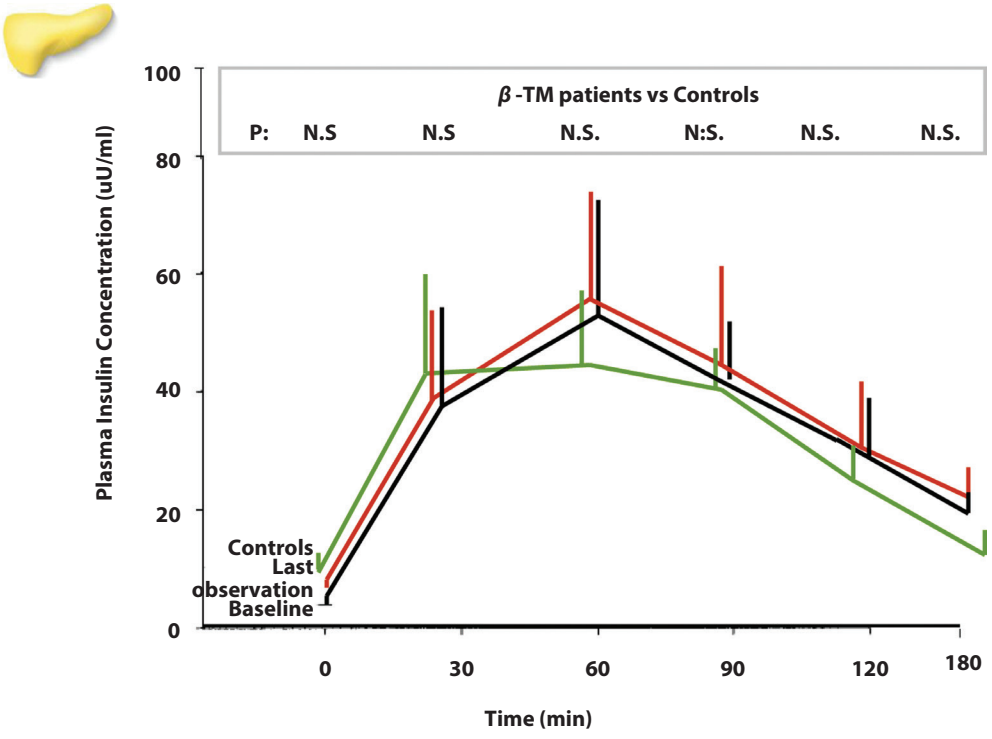

Figure 3. Profiles of insulin during oral glucose tolerance test in β-TM patients, at first and follow-up observations, and in controls.

Table 3. Significant linear correlations between insulin secretion and sensitivity, at baseline and last observation, in β-TM patients.

Insulin secretion and sensitivity indices	At first observation 11 β-TM patients	At last observation 11 β-TM patients
HOMA IR vs. Matsuda- IR Index (0-120)	r: - 0.7208; **P= 0.012**	r: - 0.7826; **P= 0.004**
HOMA-B vs. HOMA-IR	r: 0.7611; **P= 0.006**	r: 0.3589; P= 0.27
HOMA-IR vs. $AUC_{Glucose\ 0\text{-}180}$	r: - 0.0986; P= 0.77	r = 0.6794; **P= 0.021**
Matsuda IR Index (0-120) vs. $AUC_{Insulin\ 0\text{-}180}$	r: - 0.683; **P=0.020**	r: - 0.7898; **P= 0.004**
Insulinogenic index (IGI) vs. Oral Disposition Index (oDI)	r: 0.8357; **P= 0.001**	r: 0.9056; **P= 0.0001**

statistically significant correlation with HOMA-β and oDI [HOMA- β: 62.1 ± 7.5 vs. 120.0 ± 21.4 (P = <0.0001), and oDI: 4.32 ± 0.63 vs.12.11 ± 6.55 (P = 0.020)].

d. correlations

At last observation, no significant correlation was found between SF and HOMA-IR, HOMA- β,

Matsuda-IR Index (0-120), Insulinogenic index (IGI), Oral Disposition Index (oDI), $AUC_{Glucose\ 0\text{-}180}$ and $AUC_{Insulin\ 0\text{-}180.}$ The significant correlations found at first and last observation between insulin secretion and sensitivity indices are reported in table 3.

Discussion

Adult patients with β-TM are at high risk of developing impaired glucose tolerance (IGT) and thalassemia-related diabetes mellitus (TR-DM), thus an annual screening with OGTT is recommended.

The goal of this single center study was to gain further information on the OGTT follow-up, and to explore in depth the changes of β-cell function and insulin sensitivity in response to a glucose load in 11 β-TM patients with persistent low IOL, assessed by SF (range: 345-777 ng/mL).

Substantially, at last observation, the OGTT re-testing showed that 45.4% of patients had no change of glucose homeostasis, 18.1% improved, 9.0% deteriorated, and 27.2% presented a variation of their type of abnormal glucose tolerance (Figure 1). No patient developed diabetes during the follow study period.

The variability of glucose tolerance over time could probably be explained by fluctuating insulin sensitivity/ resistance, although no significant variations of indices for β-cell function and insulin sensitivity/ resistance were observed from baseline to last observation. In support of this hypothesis, in five β-TM patients, a higher insulin level at 120 min vs. 30 min was found (at first observation 0.69 ± 0.17 vs. 0.54 ± 0.11, at last observation; P = 0.77), confirming a significant dysregulation of glucose metabolism. Insulin at 30 min represents the early insulin response, whereas insulin at 120 min is mostly driven by insulin resistance (23). A low OGTT 30-min/120-min insulin ratio has been reported as a simple index for detecting prediabetes (23). However, it needs to be further evaluated in future prospective studies in β-TM patients.

An additional case of acquired HH was diagnosed in follow-up (Table 1), supporting the concept that, in spite of a low SF level (369 ng/mL), several factors affect the transport, storage and removal of iron in the different organs in β-TM patients (3,30,31). The IOL in endocrine glands develops exclusively through the uptake of NTBI. It rises considerably when the transferrin saturation is above 60%. In that case, a highly reactive Fe^{+2} subspecies of NTBI called labile plasma iron (LPI) concomitantly increases (3, 31,32). LPI enters in cells through ion transporters that are not regulated by intracellular iron concentration; thus, iron loading proceeds, even when cytosolic iron levels are very high explaining the differences of the rate of iron loading is different in different organs (30-33). The iron toxicity, due to NTBI/LPI, is mediated through the production of reactive oxygen species (ROS) (31). The primary goal of chelation is to clear the circulating ROS and to protect tissue from iron toxicity. This implies in keeping the NTBI/LPI levels in the normal range (near zero), by having circulating chelator present at all times (31). Therefore, it has been suggested that ICT should not be completely stopped even when SF is < 500 ng/mL (32).

Interestingly, no adverse events to ICT with DFO, DFX or DFP were reported in the previous years or documented at last observation. This observation confirms a previous report of Scaramellini et al. (34) in 51 out of 192 β-TM patients (32 F and 19 M, aged 44 ± 7 years) treated with DFX and SF below 500 ng/mL for at least 24 consecutive months. Based on their results, the authors suggested ICT should be continued with close monitoring of liver and kidney function tests, and IOL (34). Three out of 6 patients (27.2%), included in our follow-up study, with SF below 500 ng/dL had hypercalciuria. Two were on treatment with DFO and 1 with DFX (urinary calcium from 12 to 18 mg/kg/day; hypercalciuria was defined by urinary calcium excretion > 4 mg/kg/day in a normal 24-h urine sample).

Several authors have reported abnormalities of renal tubular function in patients with thalassaemias (35-37). The pathogenesis of hypercalciuria is not clear and several explanations have been postulated (35,37). Proximal tubular defect, due to oxidative stress associated with iron overload, and a contributory role of iron chelators could be potential explanations (36,37).

The reported rate of previous patients' OGTT screening in their own centers was quite low. Specific reasons were only partially reported. Patients' refusal to undergo the test (time-consuming nature of test, duration of test, poor acceptability, and unpleasant glucose taste) and poor adherence to the international guidelines (10) were the main reasons. These observations could suggest that alternative means of evaluation is needed for timely diagnosis of ATG in patients with thalassemia. Although, in the general population, there are other methods to screen for diabetes, such as fasting glucose, random glucose, and hemoglobin A1c, we have found that these methods are less sensitive and applicable compared to OGTT in thalassemia population (7,8,14).

Lastly, we are conscious that many centers had not had regular access to MRI for a number of patients enrolled in this study and management of ICT was mainly based on SF levels. Assessement by MRI of pancreatic IOL and pituitary morphology have been incorporated by some centers as prospective predictive markers to identify high-risk patients before end-organ damage occurs (33, 38,39), but they are not standard or routinely available and are not yet widely applied. Moreover, the high cost makes frequent monitoring prohibitive for some countries. and their relevance in adult populations is not clear as iron-mediated damage is frequently already present (40).

The strength of our study is that it includes for the first time the follow-up analysis of different markers of β-cell function and insulin sensitivity/resistance in β-TM patients with and without normal glucose tolerance test. Nevertheless, certain limitations should be recognized. These include the small number of patients enrolled in the study, the use of SF as an indirect index of body iron store and the use of surrogate indices for β-cell function and insulin sensitivity/resistance. SF is an inexpensive blood test that broadly correlates with total body iron burden, it can be measured frequently and is useful to monitor trends in iron burden over time. In β-TM patients included in this study, serial measurements of SF level (every 6 months) were collected after having excluded certain conditions that can interfere with its assessment (3,41). Previous studies have shown that Matsuda-IR index and HOMA-IR correlate well with clamp-derived measures (42,43).

In conclusion, removal of iron from the pancreas and pituitary by currently available chelators seems to be more difficult compared to other organs and highly dependent on organ-specific iron transport differences and bone marrow activity (11,12). After 8 years follow-up, despite a significant reduction of SF (at baseline mean SF: 891 mg/dL and at the follow-up:559 ng/mL) in 39 β-TM patients (aged 33-45 years), a limited effect of chelation therapy on pancreatic iron overload has been reported (11), as evidenced by the development of two new cases of diabetes mellitus and three cases of IGT (+12.8%) (11).

The lower oDI in β-TM patients with SF level below 800 ng/dL (range: 345-777 ng/dL) indicates the inability of pancreatic β-cells to compensate for the degree of insulin resistance. The ability to keep iron at near "normal" level without unacceptable risks of toxicity remains to be established. Iron chelation therapy is of paramount importance, supplemented by regular monitoring of organ iron by MRI (a LIC < 3 mg/g dry weight has been proposed) in association with clinical and biochemical parameters. Although LIC represents total body iron stores relatively well, most iron toxicity

develops in extrahepatic tissues, e.g., in endocrine organs and heart, which have different mechanisms of iron uptake and removal compared to the liver. The limitation of using SF to estimate organ iron burden has been shown in a number of studies. However, serial SF determinations may represent a surrogate index of organ iron loading when MRI is not available.

There are a number of additional questions that could not be answered by the design of our study, e.g. the effects of hypercalciuria in relation to bone densitometry and markers of bone metabolism, the changes in renal and hepatic functions of iron chelation therapy in patients with persistent SF level below 500 ng/mL, and the efficacy of different iron chelators on the pancreatic IOL assessed by MRI. Therefore, larger longitudinal studies are needed to create a more robust understanding on the indications to safely remove the excess of iron from the body.

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